Video Article

Cell-based Assay Protocol for the Prognostic Prediction of Idiopathic Scoliosis Using Cellular Dielectric Spectroscopy

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URL: http://www.jove.com/video/50768
DOI: doi:10.3791/50768

Keywords: Medicine, Issue 80, Blood Cells, Lymphocytes, Spinal Diseases, Diagnostic Techniques and Procedures, Clinical Laboratory Techniques, Dielectric Spectroscopy, Musculoskeletal Diseases, Idiopathic scoliosis, classification, prognosis, G proteins, cellular dielectric spectroscopy, PBMCs

Date Published: 10/16/2013

Citation: Akoume, M.Y., Franco, A., Moreau, A. Cell-based Assay Protocol for the Prognostic Prediction of Idiopathic Scoliosis Using Cellular Dielectric Spectroscopy. *J. Vis. Exp.* (80), e50768, doi:10.3791/50768 (2013).

Abstract

This protocol details the experimental and analytical procedure for a cell-based assay developed in our laboratory as a functional test to predict the prognosis of idiopathic scoliosis in asymptomatic and affected children. The assay consists of the evaluation of the functional status of Gi and Gs proteins in peripheral blood mononuclear cells (PBMCs) by cellular dielectric spectroscopy (CDS), using an automated CDS-based instrument, and the classification of children into three functional groups (FG1, FG2, FG3) with respect to the profile of imbalance between the degree of response to Gi and Gs proteins stimulation. The classification is further confirmed by the differential effect of osteopontin (OPN) on response to Gi stimulation among groups and the severe progression of disease is referenced by FG2. Approximately, a volume of 10 ml of blood is required to extract PBMCs by Ficoll-gradient and cells are then stored in liquid nitrogen. The adequate number of PBMCs to perform the assay is obtained after two days of cell culture. Essentially, cells are first incubated with phytohemmaglutinin (PHA). After 24 hr incubation, medium is replaced by a PHA-free culture medium for an additional 24 hr prior to cell seeding and OPN treatment. Cells are then spectroscopically screened for their responses to somatostatin and isoproterenol, which respectively activate Gi and Gs proteins through their cognate receptors. Both somatostatin and isoproterenol are simultaneously injected with an integrated fluidics system and the cells' responses are monitored for 15 min. The assay can be performed with fresh or frozen PBMCs and the procedure is completed within 4 days.

Video Link

The video component of this article can be found at http://www.jove.com/video/50768/

Introduction

Idiopathic scoliosis is a spine deformity of unknown cause generally defined as a lateral curvature greater than 10 degrees accompanied by a vertebral rotation[1]. The condition affects 4% of the pediatric population and is most commonly diagnosed between the ages of 9 to 13 years[2,3,4]. The diagnosis is primarily of exclusion and is made only after ruling out other causes of spinal deformity such as vertebral malformation, neuromuscular or syndromic disorders. Traditionally, the trunkal asymmetry is revealed by Adams forward bending test and measured with scoliometer during physical examination[5]. The diagnosis can then be confirmed by radiographic observation of the curve and the angle measurement using the Cobb method[6].

Once diagnosed, the primary concern for physicians in managing scoliotic children is whether the curve will progress. Indeed, the curve progression is often unpredictable and is more frequently observed among girls than in boys[7]. If untreated, the curve can progress dramatically, creating significant physical deformity and even cardiopulmonary problems. These manifestations become life threatening when the curve exceeds 70° [8,9]. The current treatment options to prevent or stop curve progression include bracing and surgery. In general, bracing is recommended for curves between 25-40°, while surgery is reserved for curves greater than 45° or unresponsive to bracing.

Approximately, 10% of children diagnosed with idiopathic scoliosis have curve progression requiring corrective surgery[10]. Currently, there is no proven method or test available to identify this category of patients. Consequently, all diagnosed children are subjected to multiple radiographs over several years, usually until they reach skeletal maturity. It is estimated that the typical patients with scoliosis will have approximately 22 radiological examinations over a 3-year period[11]. Because of the potential risk of multiple radiographic examinations, the alternative approaches that could allow performing the prognosis of idiopathic scoliosis without exposing children to ionizing radiation are strongly desirable. With the intention of meeting this need, we have previously developed a cell-based screening assay as a prognostic test to sooner identify the asymptomatic children at risk of developing idiopathic scoliosis. The test predicts the clinical outcome in both asymptomatic and affected children by examining the functional status of Gi proteins and classifying children into three functional groups (FG1, FG2 and FG3) according to the degree of maximum response to Gi protein stimulation[12] as measured by CDS-based system. This system is largely used to assess signal

transduction through G proteins in various cell types[13,14,15,16]. It yields information regarding the total integrated response of the cells to the external stimuli by measuring changes in impedance following the activation of cell surface receptors. Cells are seeded into microplates that contain electrodes at the bottom of the wells and the system applies a small voltage that induce extracellular and transcellular currents. Following compound injection into the well, cell surface receptors are stimulated and signal transduction events occur, leading to cellular changes that affect the flow of extracellular and transcellular currents, and thereby affect the measured magnitude. With this approach, the scoliotic patients and children more at risk of developing scoliosis are less responsive to Gi protein stimulation when compared with healthy control subjects, and the classification is based on the percentage of degree of reduction relative to control group. The classification ranges are fixed between 10 and 40% for FG3, 40 and 60% for FG2, and 60 and 90% for FG1[12].

More recently, we have modified this approach by demonstrating that the three functional groups can clearly be distinguished according to the profile of imbalance between response to Gi and Gs stimulation. Indeed, we found that response to Gi stimulation predominated in FG3, while no apparent imbalance was observed in FG2. In contrast, FG1 exhibited predominance for response to Gs stimulation. In addition, we have provided the evidence that patients belonging to FG2 are more at risk of progressing to the point of needing surgery[17]. The precision of this classification test has further been improved by demonstrating a differential effect of osteopontin (OPN) on response to Gi stimulation among functional groups.

Here we document the detailed steps of experimental and analytical procedures of this functional test as currently performed in our laboratory.

Protocol

The entire procedure is carried out under the sterile biological hood and all solutions and equipment coming into contact with cells must be sterile.

1. Preparation of Essential Solutions

1. Prepare solutions according to **Table 1.**
2. Keep balanced salt solution (BSS) at room temperature and all other solutions at 4 °C until the time of use.
3. Warm cold media to 37 °C in the water bath for a few minutes before using.

2. Preparation and Storage of PBMCs

1. Collect 10 ml of whole blood in EDTA-treated collection tubes to prepare two aliquots of PBMCs using 5 ml for each aliquot.
2. Transfer 5 ml of whole blood from the EDTA-treated collection tube to a 50 ml tube.
3. Add an equal volume of BSS and mix sample by gentle pipetting up and down.
4. Place 3 ml of Ficoll in two 15 ml Falcon tubes.
5. Carefully layer 4.5 ml of diluted blood mixture over the Ficoll in each tube.
6. Let the tubes rest for up to 5 min to favor a clear separation of the blood and Ficoll.
7. Centrifuge the tubes at 400 x g for 30 min at room temperature with no brake.
8. Carefully remove the tubes from the centrifuge so as to not disturb the layering. The PBMCs are visible at the BSS/Ficoll interface.
9. Harvest the cloudy layer of PBMCs at the interface of both tubes with a pipette and transfer to a new 50 ml tube.
10. Add 20 ml of complete media.
11. Centrifuge the tube at 288 x g for 7 min at room temperature.
12. Remove the supernatant by aspiration.
13. Resuspend the cell pellet in 500 µl of supplementary media.
14. Add an equal volume of freezing media.
15. Transfer the cell suspension to a cryovial.
16. Place the cryovial into a cryofreezing container with isopropanol.
17. Store the freezing container at -80 °C overnight.
18. Transfer the frozen PBMCs aliquot to liquid nitrogen for long-term storage.

3. Functional Assay

1. Day 1

1. Place aliquot from liquid nitrogen in water bath at 37 °C for a minute or until defrosted.
2. Transfer the cell suspension to a 50 ml tube with a sterile pipette.
3. Add 15 ml of complete media and spin the cells down at 200 x g for 5 min at room temperature.
4. Remove the supernatant by aspiration.
5. Gently suspend cell pellet in 1 ml of PHA media.
6. Complete the volume to 20 ml with the same media.
7. Cap the tube loosely to allow air to enter.
8. Leave the tube overnight at 37 °C in a CO_2 incubator to allow quiescent lymphocytes to transform into rapidly-proliferating lymphoblasts.

2. Day 2

1. Take the tube out of the incubator, screw the caps completely and spin the cells down at 200 x g for 5 min at room temperature.
2. Remove the supernatant by aspiration.

3. Gently suspend cell pellet in 1 ml of complete media.
4. Complete the volume to 20 ml with the same media.
5. Cap the tube loosely to allow air to enter.
6. Leave the tube overnight at 37 °C in a CO_2 incubator to expand cell numbers.

3. Day 3

1. Get the tube out of the incubator, screw the caps completely and spin the cells down at 200 x g for 5 min at room temperature.
2. Remove the supernatant by aspiration.
3. Wash cells twice with 10 ml of RPMI-1640 by centrifugation at 200 x g for 5 min at room temperature.
4. Gently resuspend the cell pellet in 600 µl of RPMI-1640.
5. Measure the cell concentration and viability, using an automated cell counter and viability analyzer.
6. Add appropriate volume of RPMI-1640 to adjust to a cell concentration of 1.5 x 10^5 cells/20 µl.
7. Treat cells with recombinant OPN (rOPN) or vehicle (PBS) in 1.5 ml of Eppendorf tubes.
    1. Transfer 100 µl of cell suspension to two sterile 1.5 ml Eppendorf tubes.
    2. Add rOPN in one tube to a final concentration of 0.5 µg/ml.
    3. Add an equal volume of PBS in the second tube.
    4. Gently mix each condition by pipetting up and down twice using a sterile pipette set at 100 µl.
8. Prepare the small sample 96-well microplate.
    1. Add 5 µl of RPMI-1640 to each well.
    2. Centrifuge the plate at 200 x g for 3 min to remove any air bubbles.
9. Seed the untreated cells as well as cells treated with rOPN or PBS.
    1. Before transferring cells from tube to microplate, gently pipette up and down once to ensure a uniform suspension of cells.
    2. Add 40 µl of cell suspension per well in quadruplicate for untreated cells, in duplicate for rOPN or PBS treated cells. Refer to **Figure 1** for the design. This design allows 12 patients to be tested on the same microplate.
    3. Leave the cell plate under the sterile hood for 5 min to allow cells to rest and settle evenly to the bottom of the well before placing in the incubator.
10. Incubate the plate for 18 hr at 37 °C in a CO_2 incubator to optimize the effect of OPN.

4. Day 4

1. Run the plate with compounds.
    1. Take the plate out of the incubator and leave it at RT for around 30 min.
    2. Prepare 1 ml of 100 µM of somatostatin and isoproterenol in RPMI-1640 by adding 10 µl of stock solution (10 mM) in 990 µl of RPMI-1640.
    3. Fill the compound plate by dispensing 20 µl in appropriate wells as indicated in **Figure 2**.
    4. Cover the compound plate with a precut pierceable seal to avoid change in compound concentration due to evaporation before or during incubation in the CDS-based system.
    5. Load cell plate, pipette tips, and compound plate into the CDS-based system.
    6. Name the plate in the CDS-based instrument software.
    7. Select the appropriate protocol. The protocol edited for the classification with PBMCs is called ***'Agonist Non-adherent cells Small Sample Plate RT 15 min'***. Go to the protocol box and select this protocol in the list of protocols
    8. Initiate the protocol by clicking on **'Start'.**
    9. The integrated fluidics system simultaneously adds the compounds to all wells by injecting 5 µl per well to achieve a final concentration of 10 µM in a total volume of 50 µl.
    10. The CDS-based system automatically collects the data for 15 min after compound addition.
2. Data analysis
    1. Select low and high ranges of frequencies to use when calculating extracted values for the nonadherent cells.
    2. Select drift correction to correct the linear change in baseline impedance measurements over time.
    3. Select data filtering to reduce variations in the kinetic response measurement due to electronic noise and compound addition.
    4. Select the Max-Min method for the full analysis time.
    5. Export data to Excel under the plate format option.
    6. Calculate delta G (ΔG) by subtracting the average of response magnitude to Gi stimulation (RmGi) from the average of response magnitude to Gs stimulation (RmGs) using the following formula:

        $\Delta G = RmGi - RmGs$
    7. Calculate the percentage of the fold effect (Fe) of OPN on Gi-mediated response by dividing the average of response magnitude to Gi stimulation in the presence of OPN (RmGiOPN) with the average of response magnitude to Gi stimulation in the presence of PBS (RmGiPBS) using the following formula:

        $Fe = 100 \times (RmGiOPN/RmGiPBS)$
    8. Refer to **Table 2** to classify patients.

Representative Results

Cell viability was comparable among all samples with values consistent in the range of 86 and 96%. In contrast, high variations were noted in cell numbers among samples (**Table 3**). Of the 32 samples used, two had insufficient number of cells and have not been classified. An example of results of the functional classification according to the degree of imbalance between Gi and Gs signaling is showed in **Figure 3**. The vertical axis of this figure is divided into three sections delineating the functional groups with dynamic ranges established as > +10 for FG3, between +10 and -10 for FG2, and finally < -10 for FG1. Among 30 patients tested here, 14, 6, and 5 patients were clearly classified into FG3, FG2, and FG1, respectively, while five patients, notably 345, 353, 370, 371, and 382, were at borderline of ranges. The evaluation of the OPN effect on response to Gi stimulation had revealed that OPN increased the response in patients 353 and 371. In contrast, response was reduced by more than 50% in patients 345 and 382 and by less than 50% in patient 370 following rOPN treatment. So, according to our classification criteria (**Table 2**), we were able to categorize patients 353 and 371 in FG1, patients 345 and 382 in FG2, and patient 370 in FG3. In parallel, all patients were screened for their response to Gi protein stimulation and compared to control subjects. As expected, all patients were less responsive than control subjects, and patients classified in the same functional group by our new procedure exhibited similar levels of the maximum response (**Figure 5**). Moreover, disparity between patients of each functional group was consistent with our classical range of classification [12], validating our new procedure. The classification of a large cohort of scoliotic patients regularly followed in our special clinic at Sainte-Justine Hospital has revealed that the three functional groups were similarly distributed among moderate cases, while the FG2 was predominant among severe cases (**Figure 6**), identifying patients categorized into this functional group as more at risk for severe progression of the disease and indicating that this classification test can be useful in the prognosis of idiopathic scoliosis.

Solution A	Anhydrous D-glucose	0.1%
	$CaCl_2\,2H_2O$	0.05 mM
	$MgCl_2$	0.98 mM
	KCl	5.4 mM
	Tris	145 mM
Solution B	NaCl	140 mM
Balanced Salt Solution (BSS)	Solution A	1 volume
	Solution B	9 volume
Complete media	RPMI-1640	500 ml
	Antibiotic-antimycotic	1%
	FBS	10%
Supplementary media	RPMI-1640	50 ml
	Antibiotic-antimycotic	1%
	FBS	40%
Freezing media	RPMI-1640	50 ml
	Antibiotic-antimycotic	1%
	FBS	40%
	DMSO	20%
PHA media	RPMI-1640	500 ml
	Antibiotic-antimycotic	1%
	FBS	10%
	Phytohemaglutinin	1%

Table 1. Essential solutions.

Dynamic ranges with ΔG	Functional Groups	Dynamic ranges with Fe
ΔG< -10	FG1	Fe>100%
-10 <ΔG< +10	FG2	Fe<50%
ΔG> +10	FG3	50%<Fe<95%

Table 2. Categorization of functional groups according to dynamic ranges established with ΔG and Fe.

Patients	Viability (%)	Cell concentration (×10^6/ml)	Comments
343	88.7	11.64	
344	90.5	13.6	

345	94.4	8.54	
346	94.3	25.79	
347	94.2	27.36	
348	94.6	8.52	
349	91.2	0.82	Insufficient number of cells
350	90.3	8.92	
352	92.6	8.28	
353	91.3	12.75	
354	86.9	7.62	
355	91.2	7.51	
356	90.3	9.36	
358	95.1	16.94	
359	92.3	13.89	
360	89.4	7.67	
361	93.5	7.84	
365	86.5	2.2	Insufficient number of cells
368	92.6	15.69	
369	93.4	10.9	
370	92.5	19.93	
371	88.8	10.68	
374	93.9	16.86	
376	92.9	15.67	
377	93.1	9.99	
378	93.6	13.57	
379	92.6	19.86	
380	91.1	8.46	
381	93.9	14.82	
382	92.1	23.06	
383	92.9	11.82	
384	89.1	7.73	

Table 3. Percent viability and cell concentration as determined using an automated cell counter and viability analyzer.

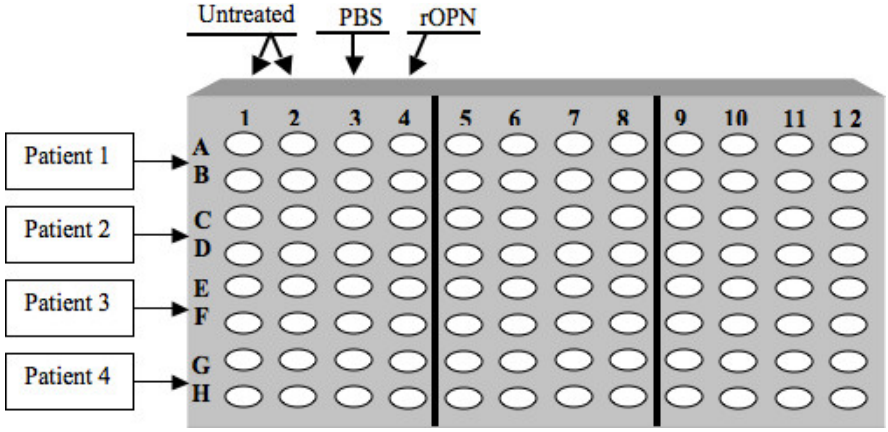

Figure 1. Design for cell seeding.

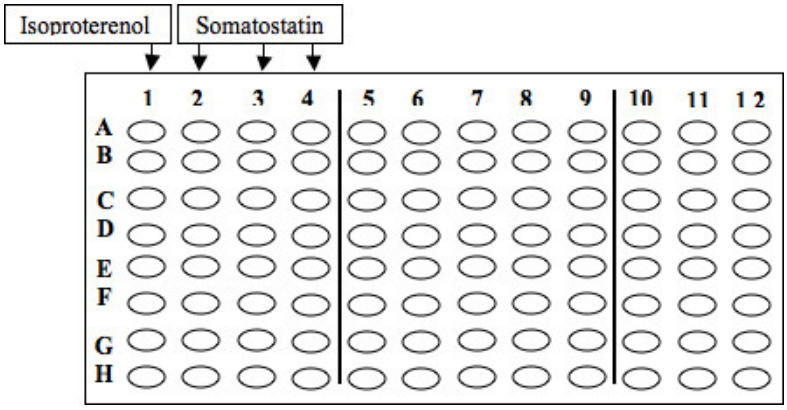

Figure 2. Design for dispensing compounds.

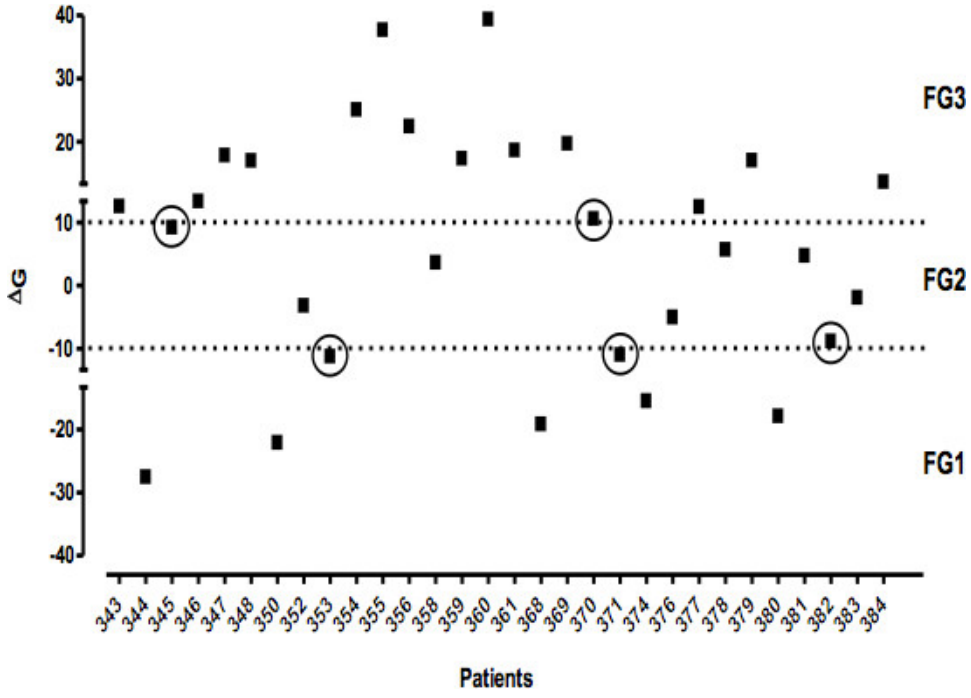

Figure 3. Dynamic range of the functional classification using the CDS-based system. Graph illustrates values of the degree of imbalance between responses to Gi and Gs stimulation obtained in PBMCs from patients with idiopathic scoliosis. Values were measured by the CDS-based system in response to 10 μM of somatostatin and isoproterenol. Each point represents the ΔG of both responses in duplicate.

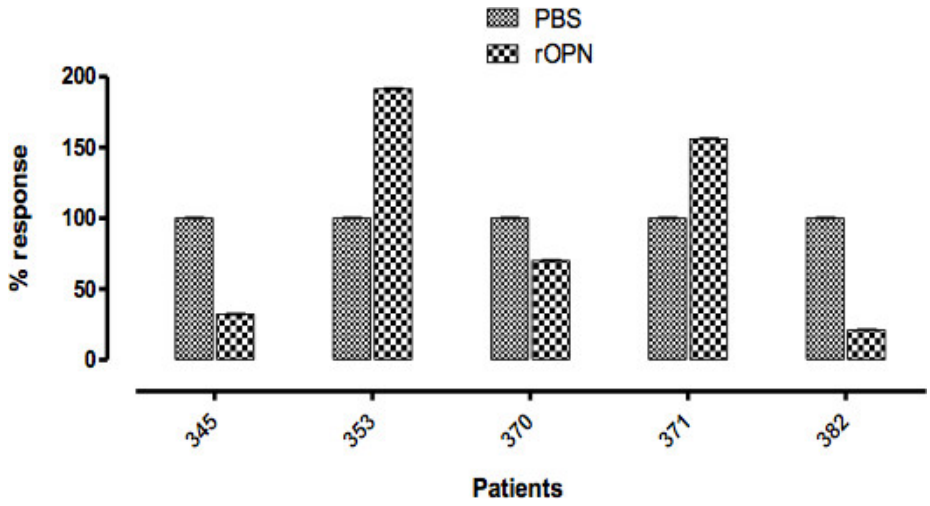

Figure 4. Effect of rOPN on response to Gi stimulation in PBMCs. Cells were serum-starved for 18 hr in the presence or absence of 0.5 µg/ml rOPN and then stimulated with 10 µM of somatostatin to initiate Gi-mediated cellular response. Data in the graph were generated from maximum-minimum impedance and correspond to the average of response in duplicate.

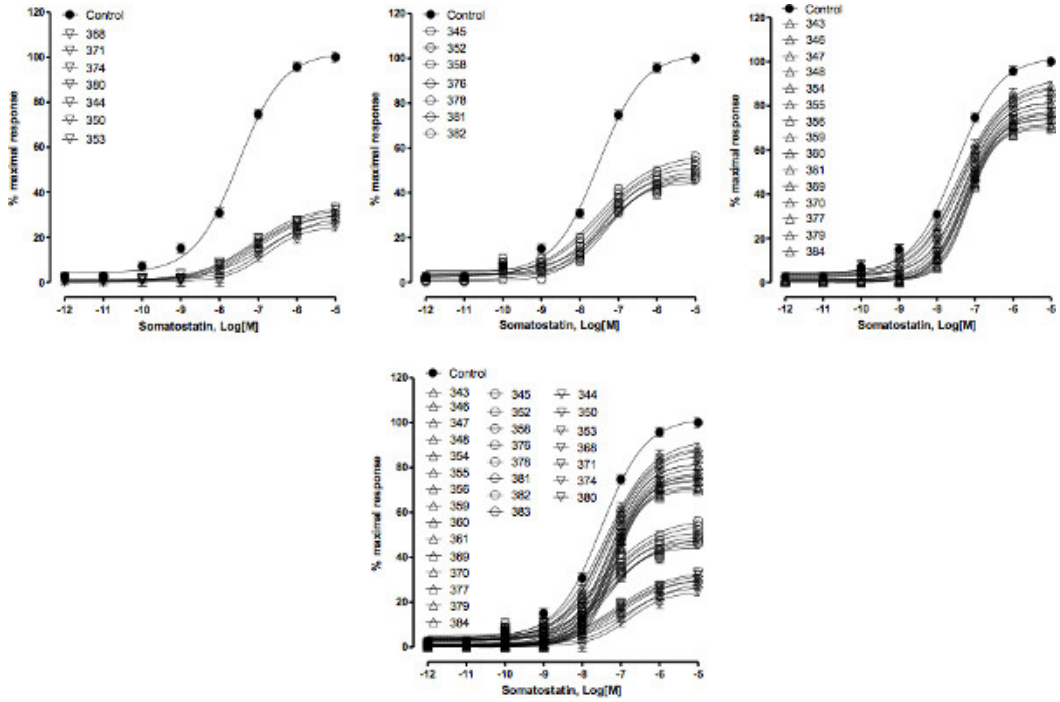

Figure 5. Functional status of Gi protein in PBMCs from control and scoliostic subjects. PBMCs from control subjects and scoliotic patients were exposed to increasing concentrations of somatostatin to stimulate Gi proteins via endogenous somatostatin receptor. The cellular response was measured by CDS-based system as described in the procedure section. Curves were generated from maximum-minimum impedance. Each curve represents the nonlinear regression. Data were normalized to maximal response in cells from control subjects and each point corresponds to the average of response in duplicate. Click here to view larger figure.

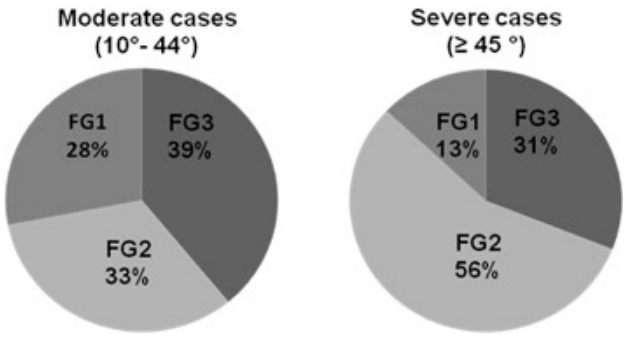

Figure 6. Distribution of functional groups among different phases of scoliosis. A large cohort of scoliotic patients with 794 moderate (curvatures between 10-44°) and 162 severe (curvature greater than 45°) cases regularly followed at Sainte-Justine Hospital, were classified according to their degree of imbalance between response to Gi and Gs stimulation. Responses were measured by the CDS-based system in response to 10 µM of somatostatin and isoproterenol.

Discussion

We have described a detailed procedure of a cell-based prognostic test for idiopathic scoliosis that is applicable to peripheral blood mononuclear cells (PBMCs) freshly isolated or conserved frozen for up to one year in liquid nitrogen. Since using freshly isolated PBMCs is cumbersome when testing large number of individuals, the procedure was presented with frozen PBMCs that offer a more practical alternative in clinical setting. However, problems with cell clumping upon thawing were encountered when frozen PBMCs were initially used, leading sometimes to inter-assay variability. To maximize assay reproducibility, we recommend avoiding freeze-thaw cycle and using the frozen sample only once. The procedure is very simple, allowing for accurate detection of defective Gi protein function in a short time. Using this procedure, asymptomatic and scoliotic children can be easily classified to better predict their clinical outcome without any danger for their health. However, when performing classification according to the degree of maximum response to Gi stimulation relative to the healthy control subjects [12], several requirements for the control subjects should be met. Indeed, in order to have a proper comparison cohort, the control subjects must be age and gender matched, not be on any kind of medication, and provide private information, such as past individual/familial medical history. These requirements may constitute an important obstacle for the recruitment of control subjects. Therefore, performing classification by examining the degree of imbalance between response to Gi and Gs protein stimulation in the same individual is ideal to eliminate the necessity of using control subjects.

The use of the CDS-based system to perform this prognostic test is significant in terms of simultaneously providing Gi- and Gs-mediated cellular responses in the same assay. Although many patients can be classified with no ambiguity using the dynamic range fixed for this assay, a small number of patients will exhibit values at the borderline of ranges, as illustrated by the results of the present report. To discriminate these individuals, we have introduced the evaluation of the effect of OPN on response to Gi stimulation by demonstrating that OPN induces a differential effect on Gi-mediated cellular response among the three functional groups. Indeed, we found that in presence of OPN, response to Gi stimulation increases in FG1, while it decreases in FG2 and FG3, to a higher extent in FG2. Despite the high cost of OPN, the use of this chemokine is essential to distinguish ambiguous cases, and therefore improve the accuracy of our classification assay. However, this assay has a disadvantage in that a minimum of 1.5×10^5 cells per well is required to observe cellular response with the CDS-based system under our experimental conditions. Certain patient samples will not have a sufficient number of cells to be tested. In this case, it is necessary to recall the patients for additional blood collection, which can be worrisome for families and frustrating for the medical and laboratory staff. Prospective studies are planned in our laboratory to address this issue.

Nevertheless, the current protocol relies on simple and proven methods to prepare cells while the testing is automated using a validated label-free system [13, 14] to monitor the cells' responses. The testing platform is relatively inexpensive when compared with genetic platform available for the prognosis of other diseases. Also, the cost of all the disposable materials, including the blood collection tubes, tips, the special electrode microplates, and conical and Eppendorf tubes, is not very expensive and is estimated at less than $3,000 to complete a testing for approximately one thousand patients. Though this estimate does not include labor costs for the preparation of samples and performing the test, this test remains considerably more affordable than next-generation sequencing platforms. Noteworthy, is not only the cost comparison to genetic platforms, but more specifically related to the field of AIS, is the costs associated to radiological imaging. Today, more than one million children in the USA and about 100,000 children in Canada are diagnosed with idiopathic scoliosis, and the total cost of diagnosis and monitoring of the scoliotic children by X-ray exposure is over 2.5 billion dollars annually in North America. Thus, our cell-based assay procedure would be expected to be suitable for routine screening and monitoring of children with idiopathic scoliosis without health risk and at lower cost.

Disclosures

This work led to several patents hold by Sainte-Justine University Hospital and many others are pending in several countries. Fourth Dimension Spine LLC is the exclusive licensee of Sainte-Justine University Hospital.

Acknowledgements

This work was supported by grants from La Fondation Yves Cotrel de l'Institut de France, Paris, France (to Dr. Moreau), The Canadian Institutes of Health Research (grant PP2-99466 to Dr. Moreau) and from Fourth Dimension Spine LLC, New York, USA (Research grant to Dr. Moreau).

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